PATENT
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In re Application of: Gregory T. Bleck, et al.
Serial No.: 10/759,315 Group No.: 1633
Filed: 1/16/04 Examiner: Riggins

Entitled: **PRODUCTION OF HOST CELLS CONTAINING MULTIPLE INTEGRATING VECTORS BY SERIAL TRANSDUCTION**

DECLARATION OF DR. GREGORY BLECK

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Dated: August 2, 2006 By: _____
Jasmine M. Stansberry

I, Dr. Gregory Bleck, state as follows:

1. My present position is Director, Molecular Biology & Transgenics Gala Biotech, a Subsidiary of Cardinal Health.

2. I am an inventor of the above referenced patent application.

3. It is my understanding that the Examiner has argued that the above patent application does not provide enablement for up to 200 retroviral integrations in a host cell.

4. Using the methods described in the application (e.g., Examples 2-5, 26-28), the production of Antibody K in Chinese Hamster Ovary (CHO) clonal cell was optimized by

retroviral integrations were obtained. The methods described in the application can be used to obtain or to 200 or more retroviral integrations.

5. In this example, the host cell lines was transduced with separate vectors containing genes encoding the heavy and light chains of Antibody K. Initially, a light chain transduction was performed, followed a week later by a heavy chain transduction. The resulting pool of cells (1X) was split. Half of the cells were used for clonal selection and half were used for the next set of transductions. A second light chain followed by a second heavy chain transduction was carried out on half of the 1X pool. Again the resulting pool (2X) was split and half of the cells were used for clonal selection and the other half used for additional transductions. A third light chain followed by a third heavy chain transduction was carried out on half of the 2X pool. The resulting 3X pool was split with half the cells used for clonal selection and half used for the fourth cycle of light chain and heavy chain transductions. The final 4X pool was also used for clonal selection.

6. The resulting four cell pools were screened to determine the gene index as outlined below. The gene index increased with each transduction as shown in Table 1. The gene index was developed using quantitative real-time PCR. For analysis of quantitative real-time PCR, an amplification plot is generated which shows fluorescence signal versus cycle number. In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of amplified PCR product. A fixed fluorescence threshold can be set above the baseline. The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Quantitative real-time PCR based assays were used to estimate the number of copies of genes inserted by GPEx™ technology into CHO production cell lines. The extended packaging region (EPR) of each gene insert is used as the target gene to estimate the total number of transgene insertions. The β 1,4 galactosyltransferase-1 gene is used as an internal control gene for the amount of genomic DNA isolated from CHO cell lines. Thus, the gene copy index generated by subtracting the EPR Ct from the control Ct reflects the number of transgene inserts that are present in the genome of the cell line. In general, gene index values are exponential – increasing by + 1 reflects ~2 fold increase of the target relative to the control.

The gene index was calculated by subtracting the Ct of the transgene assay (EPR) from the Ct for the control assay (β 1,4 galactosyltransferase-1). A higher gene copy index reflects a greater number of genes inserted into the CHO genomic DNA.

Table 1. Gene indices for each of the four transduced pools.

Pooled Population Cell Line	Gene Index
LC/HC Transduction 1	4.93
LC/HC Transduction 2	7.20
LC/HC Transduction 3	7.93
LC/HC Transduction 4	8.53

7. Each of the cell pools shown above was used for clonal selection. Clonal selection was performed as follows: 1) Limited dilution cloning was used to isolate approximately 300 clones from the transduced pooled population of cells; 2) Clones were grown in 96 well plates for 10 – 14 days; 3) The top 20 clones were selected based on protein production and were expanded; 4) Productivity assays were performed on the top 20 clones in triplicate T-150 flasks; 5) Five clones were selected for more detail analysis; and 6) The 5 clones were examined in fed-batch conditions to select the top clone. The top clone from each pool was identified and the clones were compared. Production assays in triplicate T-150 flask assays under fed-batch conditions were used to compare the four clones production (Table 2). Production of the isolated clones was higher with additional cycles of transduction.

Table 2. Production of the top clones identified from each of the four transduced pools.

Clonal Cell Line	Protein mg/L
Antibody K T1 Clone	914
Antibody K T2 Clone	1028
Antibody K T3 Clone	1376
Antibody K T4 Clone	1640

8. The high producing Antibody K T4 Clone was analyzed in more detail. Real-time PCR was used to get an accurate estimate of gene copy number for this clone. Three assays were run on this sample as outlined below. One assay detects the total number of transgenes (both light chain gene inserts and heavy chain gene inserts), another assay only detects light chain gene numbers and the third assay only detects heavy chain gene numbers. The results of the assays are shown in Table 3. The T4 clone has approximately 113 total gene inserts with ~57% of the inserts being light chain gene copies and 43 % being heavy chain gene copies.

We used a quantitative real-time PCR based assay to determine gene copy number of a cell line, in order to estimate absolute gene insert copy number in CHO production cell lines. The extended packaging region (EPR), part of antibody heavy chain gene and part of antibody light chain gene of each gene insert are used as the target genes to determine gene copy number. The β 1,4 Galactosyltransferase-1 gene is used as a single copy internal control gene for CHO cell lines. Standard curves are established using standard template DNA spiked into CHO genomic DNA background. Copy number was calculated as the number of inserts per cell, assuming 6.6pg of total genomic DNA per CHO cell.

Standard curves were generated using serial dilution of a standard DNA template for each of the three assays. The standard is diluted into CHO genomic DNA background (20ng) to mimic the matrix effect of the cellular DNA. The standards were generated using a value of 6.6 pg of total genomic DNA per CHO cell. After running the each assay for the standard samples a plot of the log of initial target copy number for a set of standards versus threshold cycle was

generated and produced a straight line. The gene copy number for the test samples were estimated using the standard curves.

Table 3. Gene copy number estimates for the Antibody K clone isolated from the 4X transduced pool.

Cell Line	Total Transgene Number	Light Chain Transgene Number	Heavy Chain Transgene Number
Antibody K T4 Clone	113	64	51

9. I further declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: July 7, 2006

Dr. Gregory Bleck